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STUDIES ON THE NON-SYMBIOTIC NITROGEN FIXATION OF SOME SASKATCHEWAN GRASSLAND SOILS

A DISSERTATION
SUBMITTED TO THE SCHOOL OF GRADUATE STUDIES
IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE
OF MASTER OF SCIENCE

FACULTY OF AGRICULTURE
DEPARTMENT OF SOILS

by

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ABSTRACT

The nitrogen-fixing powers of virgin and cultivated soils from the grassland region of Saskatchewan were studied in laboratory cultures. There was little difference in the nitrogen-fixing activity of virgin and cultivated soils inoculated into sand cultures. Aerobic conditions in sand cultures favored nitrogen fixation, indicating the importance of the aerobic non-symbiotic nitrogen-fixing microflora. Soil conditions, such as high nitrate content and acid reaction, influenced unfavorably the presence and activities of the nitrogen-fixing flora of some soils. Some soils were more efficient than others in utilizing available energy.

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INTRODUCTION

The assimilation of atmospheric nitrogen by soil microorganisms, working independently of legumes, is often disregarded when calculating the nitrogen balance of the soil. However, it is the opinion of many investigators that this form of bacterial activity plays an important part in maintaining soil productivity.

In 1885, Berthelot demonstrated that soil organisms could assimilate gaseous nitrogen. Winogradsky in 1893 finally isolated an anaerobic form which he called Clostridium pastorianum. Two aerobic forms were isolated in 1901 by Beijerinck, to which he gave the generic name Azotobacter.

Up to the present time, the aerobic non-symbiotic nitrogen-fixing organisms have received most attention and are often considered to be the more important. Because of the economic importance of nitrogen fixation in the maintenance of soil fertility, keen interest has been taken in the study of these organisms. The literature is filled with reports concerning the occurrence of these bacteria and factors affecting their distribution and activities.

To the writer's knowledge, there has been very little, if any experimental work on the non-symbiotic nitrogen-fixing processes in Saskatchewan soils. Being the largest

Introduction

grain producing province in Canada, Saskatchewan embodies a wide range of soil and climatic conditions.

This report is the result of preliminary studies on the nitrogen fixing flora of some Saskatchewan soils. The three grassland soil zones, brown, dark brown and black, comprise the main farming area. The investigation reported here is concerned with the soils from this grassland region.

Of the total calculated area of the Saskatchewan grassland region of 37.5 million acres, 30.5 million acres are shown by Mitchell et al. (62) to be arable. The non-arable lands are mainly grazing areas.

Nitrogen is not generally a limiting factor in crop production as shown by fertilizer trials in these zones. What part then does atmospheric nitrogen-fixation by non-symbiotic soil bacteria play? It is well known that cropping removes considerable nitrogen in grain and straw. A classical figure often used is 60 pounds of nitrogen removed by a 30 bushel wheat crop.

Legumes are not responsible for returning very much nitrogen to the soil in this region because of the dry conditions which limit their use.

Inasmuch as maintenance of a proper forage cover on the grazing lands is of importance to the stockman, virgin soils as well as cultivated soils were sampled in the study reported here.

It was considered probable that climatic and other conditions might have exerted an influence on the microflora

of these regions. One would expect in these grasslands, that an equilibrium has been reached between the soil, climate, soil organisms and higher plant life. This probably would be even more evident in the semi-arid region where vegetation is of a climax type.

It is very difficult to determine the amount of nitrogen fixed by non-symbiotic bacteria in the soil under field conditions. Various workers have estimated the amount of nitrogen fixed at from 15-40 pounds per acre per year. If the organisms responsible were inactive in Saskatchewan grassland soils, it would be of importance to determine what factors are detrimental to their growth.

It was hoped in this investigation to determine the presence and activity of the aerobic, non-symbiotic, nitrogen-fixing flora under laboratory conditions. When mentioning nitrogen fixation in this report, unless otherwise noted, the aerobic non-symbiotic form of nitrogen fixation is referred to.

REVIEW OF LITERATURE

The literature on non-symbiotic nitrogen-fixation in the last 30 years has been abundant and it would be impossible in this thesis to cover more than a small portion. A fairly complete review of the subject would be desirable, however, in order to better interpret results of this investigation.

Distribution of Nitrogen-Fixing Organisms

The presence of soil microorganisms capable of fixing atmospheric nitrogen non-symbiotically, has been reported from many parts of the world, comprising a wide range of soil and climatic conditions. Gainey (29) estimates that half of all soils examined in Kansas contained azotobacter. Martin (58) surveying reports from workers all over the world found that, of 6,257 soils examined, 3,022 or 48% contained azotobacter. In a general survey of Szechuei province, China, Gaw (32) found the aerobic nitrogen-fixing organisms were present in 78 percent of all soils examined.

In a review of the subject, Smith (73) reports Chang as having isolated azotobacter from practically all soils of Manchoukuo province in China. Sushkina of Russia was reported by Smith (73) as unable to isolate the organism from virgin soils of the arid regions; but after irrigation of these soils, azotobacter appeared. Smith (73) found that Finland was the only country reporting which had not been able to isolate azotobacter.

Ziemiecka (91) found the nitrogen-fixing organism in most soils of Poland. In his comprehensive review, Waksman (85) stated that the organism had been isolated from soils of Egypt, Africa, Italy, Australia and many other countries. Nitrogen fixers were reported by Ohmasa (66) to be present in 8 out of 29 soils from the forests of Tokyo University in Japan. Altson (4) found strains of the organism present in

certain Malayan soils where the pH was above 6.0.

The foregoing review has indicated a wide distribution of the azotobacter regardless of climatic and soil variations.

Numbers and Activity in Different Soils

While the azotobacter organisms are widespread, their numbers and nitrogen-fixing powers in various soils are not always similar. A number of workers have compared virgin and cultivated soils in this respect.

Greaves (37) examined hundreds of soils from Utah. He found that numbers and fixing powers of the bacteria were twice as high in cultivated soils as in virgin soils. In a study of western Kansas soils, Gainey (29) found azotobacter were seldom present in virgin, but abundant in culvitaved soils. Joffe (48) states that the virgin soils of Crimea showed little azotobacter activity, while those present in cultivated soils were quite active in fixing free nitrogen. He considers that failure of these soils to respond to nitrogen fertilizers is due to this active nitrogen-fixation.

Martin (58) reported that of 94 cultivated samples from Arizona, 82 contained the Azotobacter. Of 119 virgin soil samples from the same area, only 27 contained these microorganisms. In laboratory culture studies he found 19.6 milligrams of nitrogen fixed per gram of cultivated soil and only 5.3 milligrams of nitrogen fixed per gram of virgin soil. Martin and Ayers (59), also working with Arizona soils in the

laboratory, found that: (1) 67 out of 76 cultivated soils contained the azotobacter organisms; the other nine soils were high in salt; (2) those cultivated soils containing azotobacter, fixed 22 milligrams of nitrogen per gram of soil; (3) only 6 out of 23 virgin soils contained azotobacter and these showed an average fixation of 0.3 milligrams nitrogen per gram of soil.

Greaves and Bracken (37) inoculated soils from Juab and Cache counties into synthetic culture media. Cache county soil fixed more nitrogen, they indicated, because of the higher content of carbonaceous material, and also a higher initial azotobacter population.

The nitrogen content of virgin semi-arid soils of Kansas is quite low, according to Gainey (28). He considers this due to inactivity of the nitrogen-fixing flora. The nitrogen level decreases upon cultivation until a level of about 0.1 percent is reached. He suggests that increased activity of free living nitrogen fixers after cultivation prevents the nitrogen from being depleted entirely.

Vandecaveye and Moodie (83) working with eastern Washington soils, observed that: (1) there was no difference in the azotobacter population of virgin and cultivated soils in non-irrigated areas; (2) azotobacter were present in abundance in the irrigated soils of the 10 inch rainfall belt; (3) they were frequently present in the non-irrigated soils of the 15-20 inch rainfall belt; (4) they were rarely present in the non-irrigated soils of the two low rainfall belts, that is, where rainfall was less than 15 inches.

Other Nitrogen Fixing Processes

Not all aerobic nitrogen fixation reported is due to azotobacter. It might be well to mention briefly a few claims for aerobic nitrogen fixation attributed to other organisms and phenomena.

Green algae were at one time credited with fixing nitrogen in the soil, but Allison and Morris (2) found that this was not the case; neither could they demonstrate any symbiotic relationship between the azotobacter and green algae. They did show fixation by Anabaena and Nostoc, two genera of the blue-green algae. Stokes (77) could not obtain nitrogen fixation by using mixed cultures of azotobacter and green algae. The algae, he said, did not secrete enough energy to promote nitrogen fixation by the azotobacter.

Fungi and bacteria have also been credited with fixing some nitrogen. Carter and Greaves (15) found that the nitrogen fixing azotobacter were present in most Utah soils and fixed atmospheric nitrogen. However, they found one soil on the Nephi experiment station which contained no azotobacter, but fixed nitrogen actively. They isolated 27 organisms, 26 of which fixed nitrogen. Present were 10 actinomyces, 8 bacilli, 8 micrococci, and 1 penicillum.

Krishna (50) reported fixation of nitrogen by fungi. Lipman (53) obtained fixation of nitrogen by yeasts and fungi. Stoklasa, whose work is reviewed by Greaves (36), found that azotobacter were especially abundant where blue-green algae were present in the soil. He did not elaborate.

A few investigators have suggested that nitrogen increases have come about by other than microbiological activities.

Ingham (46) discounts nitrogen-fixation by non-symbiotic organisms. His reasoning is that: (1) combined nitrogen is present and evidence indicates that 10 parts per million nitrate or ammonia nitrogen inhibits fixation, (2) large amounts of energy are required (100 pounds of organic matter for every pound of nitrogen fixed) and this would not be available in the soil. He found that cellulose filter paper absorbed atmospheric ammonia and says that cellulose in the colloidal complex is responsible for soil nitrogen increases. He says that artificial fertilizers are still necessary to supplement the soil nitrogen supply.

A number of agricultural workers in India have attributed nitrogen gains to photochemical fixation. Dhar et al. (19) reported extraordinary gains due to the effects of light. They reported that: (1) soils rich in energy fixed more nitrogen when exposed to light than in the dark, (2) there were less azotobacter present in cultures kept exposed to light. Nitrogen fixation was considered as due to solar light.

Factors Influencing Nitrogen Fixation

In this review, we will discuss factors which may have influenced the nitrogen fixing flora in soils of the region sampled for this project.

(1) Effect of Reaction

There is a fair amount of agreement evident in the literature on this phase of the subject.

Fred and Davenport (24) found the azotobacter to be much more sensitive to changes in reaction than the legume bacteria. They used a nitrogen-free solution and found the critical acid value for these organisms to be pH 6.5 and the alkaline value pH 8.6. The critical acid value was found by Gainey (27) to be pH 6.0. This agrees with most findings as to the lower pH limit at which the bacteria are active.

Burk et al. (14) showed that nitrogen fixation ceased at a pH below 6.0. However, when combined nitrogen was present in the medium, the organism could survive a pH as low as 4.0, by using this form of nitrogen.

Altson (4) isolated a few strains of azotobacter from Malayan tropical soils which tolerated reactions as low as pH 3.6. The strains were not active, however, when inoculated into suitable media.

In soils of Japan, Ohmasa (66) found azotobacter where the pH was higher than 6.0 but not within the pH range of 4.0-5.5. Martin (58) could not isolate the nitrogen-fixing organisms from mountain-meadow soils of Arizona, where the pH range was 4.9-6.2. Starkey and De (75) isolated a new species which they named Azotobacter indicum. It was capable of fixing atmospheric nitrogen at reactions ranging from pH 3.0-pH 9.0.

The limiting reaction on the alkaline side for nitrogen

fixing organisms has been shown by Martin (58) to be between pH 8.8-9.2. Burke and Lineweaver (14), also reviewed by Stephenson (76), carried on respirometer studies with pure cultures of azotobacter organisms. Their studies indicated optimum growth at pH 7.6-7.8 with both free and combined nitrogen.

A few observations should be made regarding the above review.

1. Some of the work was with laboratory solution cultures and often pure cultures of the organisms were used, thus differing from field conditions.

2. Measurements made on solutions, or on ration of 1:5 of soil to water give pH readings about 1.5 pH units higher than when pH measurements are made at the moisture equivalent moisture condition according to McGeorge (61).

It is conceivable then that reaction limits, found in present day studies by soil paste pH measurements, may vary from those reported in earlier literature.

(2) Effect of Individual Ions.

Considerable research has been reported regarding the part played by various elements in fixation of nitrogen by azotobacter.

Some workers have shown these organisms to be sensitive to deficiencies in soil phosphorus and calcium. The well-known azotobacter (plaque) test for phosphate deficiency is dependent on the sensitivity of azotobacter to deficiencies in phosphorus.

Greene (41) concludes that the test is useful only where soil is extremely deficient in phosphorus. He also reasons that since organisms can adapt themselves to such differences as high temperatures, etc., they can probably do likewise with low concentrations of nutrients. He cites Stoklasa's research, which indicates that 5.0-5.7 milligrams of nitrogen are fixed for every gram of phosphorus used. The minimum required was found to be 2.46 milligrams of phosphorus per gram of glucose consumed. Greene (41) observed from his own results that there was: (1) nitrogen fixation when 1-2 milligrams phosphorus were present per 100 ml. of solution, (2) maximum fixation when 1.75 milligrams were present per 100 ml. of solution.

Ziemiecka (91) in Poland, suggested that non-symbiotic nitrogen fixing powers of soils were an index of pH and lack of soluble phosphate. It was shown that: (1) lack of soluble phosphate prevented fixation of nitrogen but the organisms were present, (2) the organisms were inactive in the range 0-10 parts per million of soluble phosphate, (3) phosphate additions increased the activity of the azotobacter in the above soils.

In Indiana, Roberts and Olson (70) applied phosphatic and potassium fertilizers and obtained increased yields. When inoculated into mannite solutions, the fertilized plot soils failed to show increased flora or activity over similarly inoculated cultures from check plot soils. Katznelson (49)

inoculated azotobacter into variously treated soils. Phosphate fertilization of soils tended to enhance ability of the soil to support the organisms. Martin (58) found that phosphate contents of Arizona soil samples were not correlated with the activity of azotobacter even when the available phosphorus content was as low as 1 part per million. This substantiates findings of Vandecaveye and Anderson (82) who found phosphorus additions non-stimulating to the azotobacter in Iowa and Washington soils, when applied even in large quantities. Burk and Lineweaver (12) in pure culture work also showed phosphorus to be required in only minute quantities.

It would appear that phosphorus deficiency can be a limiting factor for azotobacter activity in some soils. This would possibly be true in areas of highly alkaline calcareous soils, where phosphates are often unavailable to plants.

Most artificial media for study of azotobacter organisms include calcium carbonate. Gainey (27) has shown that liming acid soils to a pH above 6.0 will transform them into suitable habitats for the organism. In further work (31), he introduced a calcium clay into regular Ashby's medium, at two buffered pH levels. He found that azotobacter were active above pH 6.0 but not below pH 6.0 where calcium was available from the colloidal clay. He concluded that lack of calcium was not the factor responsible for failure of azotobacter to fix nitrogen below pH 6.0.

Burk and Lineweaver (13) using the Warburg respirometer technique, showed that calcium or strontium is essential to

nitrogen fixation in pure cultures. The bacteria could grow without calcium, however, with combined nitrogen present in the medium, if magnesium or barium were also present.

Martin (58) showed by statistical treatment of his analysis, that: (1) the water soluble sodium and calcium contents of the soil were closely related to azotobacter activity, (2) there was little correlation with soluble magnesium and phosphate contents of the samples, (3) chloride and sulfate were correlated only by reason of their relationship to calcium and sodium, (4) the maximum limit for fixation in presence of sodium and calcium was around 3,000 parts per million.

(3) Effect of Alkali Salts

This phase of the literature is possibly the most controversial of all. While alkali is not a serious problem in the areas sampled in this project, increased irrigation may produce such problems in some areas of Saskatchewan.

Greaves and Lund (39) consider that toxic effects of soluble salts on bacteria and plants are due mainly to osmotic effects on the cell. Greaves (38) found that: (1) most salts toxic to plants are also toxic to bacteria and at lower concentrations, (2) the same order of toxicity of various salts obtained, (3) smaller amounts of salts stimulated nitrogen-fixation, (4) nitrogen-fixing organisms remained active at higher salt concentrations than other groups, such as nitrifying and ammonifying bacteria.

Burgess (11) found active nitrogen fixation up to 3.0% concentrations of salts in soil and solution cultures. Martin (58) reviewed the early work of Lipman and Sharp, who found the following as maximum concentrations in the soil solution for azotobacter activity: (a) sodium chloride - 0.5%, (b) sodium sulfate - 1.25%, (c) sodium carbonate - 0.4% percent.

Lipman and Burgess (55) showed that: (1) marked differences were obtained when calcium carbonate and magnesium carbonate were applied in large quantities, (2) calcium carbonate was stimulating, never toxic, up to 2 percent, (3) magnesium carbonate was toxic above 0.1-0.2 percent, (4) in mixtures they were less toxic since the calcium carbonate was considered to exert a protective action in both soil and solution cultures against the toxicity of the magnesium.

Nitrogen Relationships

The nitrogen present in the soil in various forms exerts a profound effect on the nitrogen-fixing process. The nitrate form of available nitrogen is usually present in fallow soils under favorable moisture conditions.

Winters (90) found with soil cultures in the laboratory that: (1) nitrogen fixation was stimulated by adding 50-100 parts per million sodium nitrate or calcium nitrate, (2) 500 parts per million sodium nitrate decreased nitrogen fixation, (3) rapid nitrogen fixation was at first shown to reduce the nitrate content of soil, indicating that the organism assimilated some of it.

Gainey (30) found that: (1) relatively high concentrations of sodium nitrate (such as is often found in fertile soils) tended to depress nitrogen fixation and azotobacter population, (2) the decrease varied with different soils, (3) the sensitivity is due to the nitrate ion and not the sodium or calcium, (4) when urea was added, nitrogen fixation was depressed only after nitrification of the urea took place.

Aso et al. (5) studied the effects of different forms of nitrogen on azotobacter activity. Using Ashby's (3) culture solution, with added sodium nitrate, a trace of nitrite and ammonia was found in solution. They concluded that azotobacter use nitrate or nitrite nitrogen only after conversion to ammonia.

Seasonal variations in nitrate content of the soil have been reported by many workers. Roberts and Olson (70) found that the high concentrations coincided with lowered nitrogen fixation. Zoond, as reviewed by Thompson (78), found that concentrations over 30 p.p.m. of nitrate nitrogen, amino nitrogen, or peptone in mannitol solution, depressed nitrogen fixation.

Fuller and Rettger (26) tested effects of various nitrogen compounds and found that non-toxic materials did not influence nitrogen fixation appreciably. Horner and Allison (44) agreed with the above as regards most soils that they studied.

Thompson (78) inoculated different soils with azoto-

bacter and found low fixation where the nitrate nitrogen content was high.

Wilson and Burris (88) working with pure Azotobacter chroococcum cultures, and isotopic nitrogen (N^{15}) found that: (1) ammonia was readily accepted as a source of nitrogen to the exclusion of atmospheric nitrogen, (2) nitrate or nitrite nitrogen did not inhibit atmospheric fixation until converted to ammonia, (3) this required a period of adaptation in culture and it is suggested that the bacteria produce enzymes which convert the nitrate nitrogen to ammonia nitrogen.

Combined nitrogen in the medium was shown by Burk et al. (12) to contribute to survival of the organism under adverse conditions. If the pH is low, for example 4.0, the organism uses the nitrogen in the medium for cell growth.

Efficiency of nitrogen fixation was shown by Hills (43) to decrease upon adding available combined nitrogen. Numbers of azotobacter increased but amount of nitrogen fixed did not increase proportionately.

Burk et al. (12) working with pure cultures in solution found that: (1) concentrations of 50 p.p.m. combined nitrogen retarded nitrogen fixation, (2) the nitrogen composition of the dried cells remained at 2% regardless of the source of nitrogen.

It would appear from the above review that the azotobacter are not unlike many other organisms in that they choose the line of least resistance in obtaining their food.

Seasonal Effects

Using laboratory cultures, Batchelor and Curie (6) noted a gain in nitrogen, then a loss and another gain, when cultures were analyzed periodically. They concluded that a single incubation period does not give a true picture. Using silica-gel plates, Vandecaveye (81) made periodic counts of azotobacter on field samples for seven years. He found: (1) a marked seasonal fluctuation, and (2) a gradual disappearance of the organism from some soils. Roberts and Olson (70) noted seasonal effects on numbers and nitrogen fixing powers. Bortels (8) stated that immediate weather conditions, such as barometric pressure, moisture, temperature and light, influenced nitrogen fixation. He claimed that the higher pressures cause the formation of condensation products which inhibit nitrogen fixation.

Temperature and humidity have been indicated as being important to azotobacter growth. Ashby (3) in his early work, noted that: (1) nitrogen fixers are confined to the upper few inches of soil in humid regions, (2) they are quite active to a depth of 3-4 feet in arid soils. Aeration and temperature effects are probably responsible for these differences.

Burgess (11) working with desert soils of Arizona found that: (1) aerobic nitrogen fixation is active below moisture contents optimum for plants, (2) it is especially rapid upon alternate wetting and drying of the soil, (3) favorable temperature to the microflora was 30-40°C.

Energy Relationships

A large number of energy materials may be utilized by the azotobacter, but they vary markedly in their ability to promote the nitrogen fixing process.

Fedorov (23) found that additions of straw in the presence of 0.5% calcium carbonate: (1) decreased yields in the first year, (2) increased yields in the second year by 273%, (3) increased yields in the third year by 140%, and the nitrogen balance was not lower in the third year than it was in the control. He concludes that straw induced nitrogen fixation rather than denitrification.

Winters (90) found that manures were most valuable in promoting fixation. The soils used were low in pH, so that improved soil reactions possibly were partly responsible.

Materials such as green manures and crop residues, Greaves (35), cellulose, Krishna (51), sugars, Smith (73), organic acids, Reuszer (69) and a host of other energy materials have been used to promote nitrogen fixation. Many of these are not practical under field conditions.

Brown and Allison (10) found that the C:N ratios were of no value in indicating the effects of organic materials on the nitrogen fixing process. Martin et al. (60) found the activity of azotobacter in some Iowa soils to be closely dependent upon organic matter content, other factors being favorable.

Winogradsky (89) concludes that the only nutrients available for the use of azotobacter in its natural soil

environment are the waste products of organic matter decomposition, such as cellulose, hemicellulose, lignin, organic acids and alcohols.

Association with Other Microorganisms

The preceding review brings up the important question of the effects of other soil microorganisms on the azotobacter population. Many organisms break down organic matter and also use soil nutrients. It seems logical, then, that they would compete with the nitrogen fixers for available nutrients and energy material.

Katznelson (49) wondered about the small population or absence of azotobacter in some soils. By inoculating the organism into variously treated soils he found that: (1) there was an increase in number, only when a readily available source of energy was added, (2) under apparently favorable conditions, failure of azotobacter to increase was due to (a) unfavorable competition with other microorganisms, (b) the presence of toxic substances, and (c) absence of nutrients such as phosphates.

Vandecaveye and Villaneuva (84), using soil cultures with available nitrogen present, noted that: (1) the fungi and bacteria were first to use the available organic material originally present or added, (2) activity of the azotobacter population became more intensive after the available supply of organic material was used up. This indicated that the

nitrogen fixers used the by-products of other bacteria and fungi as energy, and agrees with Winogradsky's (89) conclusions as noted earlier in this review.

Lind and Wilson's (52) results showed that azotobacter fixed more nitrogen when cultured with other microorganisms, than when in pure culture. Possible reasons suggested are: (1) liberation by the microorganisms of essential elements such as iron and molybdenum, (2) the production of superior sources of carbon, alcohols, fatty acids, etc., (3) assimilation of ammonia which, if present, retards nitrogen fixation. It is suggested that azotobacter supplies accessory growth factors to other organisms.

Jensen (47) showed that nitrogen fixation took place in cultures of azotobacter associated with cellulose decomposing organisms. This was not the case where actinomycetes were associated with azotobacter. The cellulose by-products apparently serve as energy for the nitrogen fixers.

Not all associations with other microorganisms are beneficial. Nickell and Burkholder (64) used a culture medium containing soil, fertilizers, and organic matter. They found by culturing azotobacter and actinomyces on this medium that: (1) there was inhibition of nitrogen fixation by azotobacter, due to substances produced by the actinomyces, (2) azotobacter numbers were reduced or the organisms completely disappeared.

Rhizophere Effects

The association between soil microorganisms and plant

roots has received some study in recent years. Since we are also studying virgin soils in this project, it would be desirable to review this subject briefly.

Lochhead (56) found bacteria much more active physiologically in the rhizosphere and suggested that roots may excrete biotin, thiamin, etc., which are beneficial. Allison (1) gives an excellent review of investigations carried out in Russia by inoculating azotobacter into the soil. Claims of larger crop yields have been reported, but most work since has not substantiated these claims. He concludes that: (1) there is no proof that azotobacter thrives in the rhizosphere, (2) there is no evidence that root excretions provide energy for much fixation, (3) where residues are present, energy must first be made available by other microorganisms that use considerable for their own activities.

Allison (1) states that plant growth is not increased by azotobacter in the rhizosphere. He indicates the possibility that growth-promoting substances produced by azotobacter may be responsible for the increased yields that have been reported in Russia.

Reuszer (68) says that nitrates are generally low in pasture soils but fluctuate in cultivated soils. This is apparently due to assimilation of nitrates by the plants and the lower moisture conditions which reduce nitrification.

Newton et al. (63) made extensive studies on nitrification under and after various grasses. They found that the nitrate content of grass and alfalfa plot soils was generally lower than that

of the wheat plots, especially in drier seasons.

Hall (42) found that South African virgin grassland soils never contained more than 3.0 p.p.m. of nitrate nitrogen at any time during the year. Eighteen days after plowing virgin soils, they contained as high as 40 p.p.m. of nitrate nitrogen. A low nitrate habitat should be favorable to nitrogen fixation, other factors being suitable. Reuszer (68) obtained no significant differences in total numbers of bacteria between virgin and cultivated soils using the plate count method of determination.

Laboratory Studies

Most studies on the fixation of atmospheric nitrogen have been carried on in the laboratory. Naturally one must use caution in interpreting these results in terms of field conditions; nevertheless, these methods have an application in the study of nitrogen fixation.

Turk (80) used soil cultures, solution cultures, agar and silica-gel plate methods for studying various soils, and the effects of plot treatments in the field. He found: (1) a correlation between soil treatments, when compared in solution and soil cultures, (2) that amounts fixed were of a different order, (3) that there was no correlation between numbers of colonies on plates and nitrogen fixation, (4) there was no relation between numbers of colonies produced on the two different types of plates.

Greaves (36) noted: (1) that fixation was greater

where the culture medium was soil than where solution, (2) that there was no correlation of results between the two methods and different soil samples. Greaves and Pulley (40) added a soil extract to the usual Ashby's medium. Fixation was always greater where soil extract was present, indicating some substance in the soil favoring growth of the organisms.

Because liquid and agar media differ considerably from soil media, Madhok (57) developed a new one by adding a colloid. He claims that inorganic and organic colloids will carry the essential elements. Soil after sterilization was unsuitable, he says, as a culture medium. He used a mixture of sand 97.0%, bentonite 2.5% and humus 0.5 percent. He also used sand alone, and sand 97.5% plus bentonite 2.5 percent. The sand-bentonite mixture was found most suitable for most microbial processes. Prolonged sterilization of the sand-bentonite mixture caused reduced numbers and nitrogen fixation was lowered. Sand alone produced as high fixation as the other mixtures, but in these experiments the incubation time was 5 weeks, which may mask indications of rates of fixation. All of the above had nutrients added.

Conn (16) found that changes in the colloids of soils when air-dried made them unsuitable as culture media for about two weeks after being moistened. Conn, H.J., and Conn, J.E. (17) searched for a synthetic medium to replace soil. They decided on a mixture of sand 9.5 gms., colloids 0.5 gms., glucose 1% plus water, in test tubes.

They obtained higher counts in this mixture than in soil, and nitrogen fixation was also higher in the mixture. Sterilizing for more than an hour caused lower counts in the sand-bentonite mixture.

Curie (18) compared a few laboratory methods for studying nitrogen fixation and pointed out their limitations as follows:

(1) Soil tumbler method: This was unsuitable because of low amounts fixed and available methods are not suitable for measuring small amounts of nitrogen.

(2) Solution methods: (a) aerobic conditions are found only at the surface; (b) butyric acid is frequently found, which limits azotobacter growth.

(3) Plate method: (a) there is variation in moisture content on the surface of plates, (b) silica-gel plates are hard to prepare. He did find that numbers of colonies growing on nitrogen-free plates were a function of the nitrogen fixed per plate.

Gainey (28) presents data showing that when soil is inoculated onto agar plates: (1) the rate of nitrogen fixation is proportional to the number of colonies, (2) the quantity of nitrogen fixed (with ample incubation) is independent of the number of colonies of azotobacter growing.

Hunter (45) bubbled sterile air through the culture media. He found: (1) that fixation was speeded up by aeration, (2) also that calcium carbonate was not essential to pure cultures undergoing vigorous aeration, (3) a close correlation between

dextrose consumed and nitrogen fixed.

Waksman and Starkey (86) reported Lipman's work, which showed that azotobacter uses mannite more efficiently when in smaller concentrations. This indicates that nitrogen fixation should be highly efficient under field conditions. Turk's (80) results show that the rate of fixation is a function of number of organisms, but quantity fixed is ultimately dependent on available energy, excluding variations due to differences in microflora, toxic effects, etc.

Greaves (33) said that quantities of nitrogen fixed in culture medium vary with the number of organisms inoculated into it.

Anaerobic Nitrogen Fixation

The reader will have noted that the aerobic nitrogen-fixing process was stressed above. It has not been intended to suggest that the aerobic process is all important to the exclusion of the anaerobic one. Many workers have considered the anaerobic nitrogen-fixing process as the more important of the two in the soils which they studied.

As noted earlier Winogradsky isolated Clostridium pastorianum, one of the organisms of the Clostridium genus which fix atmospheric nitrogen anaerobically. Waksman (85) in reviewing Winogradsky's work, shows that anaerobic nitrogen fixing organisms have similar basic requirements to azotobacter. These are: (1) presence of suitable energy, (2) a source of atmospheric nitrogen. They are both limited in fixation by

combined nitrogen, and presence of calcium carbonate favors their growth. The Clostridium pastorianum has an optimum pH range of 6.9-7.3 but is active at pH 5.7. The organism has been reported from soils all over the world. Rather interesting and possibly significant is the fact that the clostridia are obligate anaerobes, but will survive under certain conditions where aerobic bacteria are present. For this reason, a symbiotic association of azotobacter and clostridia in the soil has been suggested by Lind and Wilson (52). The azotobacter, using oxygen, tend to produce anaerobic conditions for the clostridia, which by their growth would produce organic acids, etc., as energy for the azotobacter.

In laboratory studies with Michigan soils, Turk (80) concluded that the anaerobic nitrogen-fixing process was more important than the aerobic one. He reached this conclusion because the anaerobic organisms were more widespread in Michigan soils. Azotobacter when present, however, fixed more nitrogen than the anaerobic clostridia. Burgess (11) using irrigated arid soils, found in the laboratory that the Clostridia were present and quite active. He obtained fixation of from 1.4-4.3 milligrams of nitrogen per gram of mannite consumed.

It is evident from the above that the anaerobic organisms can be important contributors of soil nitrogen where present and active in the soil. The aerobic type of fixation has been stressed in this review, because conditions were made more suitable for aerobic growth in the project reported here.

It is evident that little if any work has been carried out on the non-symbiotic nitrogen-fixing process as applied to Canadian prairie soils. Claims for nitrogen fixation as reported in this review, must always be tempered by a consideration of the methods used and existing soil conditions.

Laboratory methods do, however, provide a means of showing the presence or absence of the organisms responsible, and the relative activity of the nitrogen fixing flora found in different soils.

OUTLINE OF INVESTIGATION

The present work was undertaken to determine the distribution and activity of the non-symbiotic nitrogen-fixing bacteria in some soils of the Saskatchewan grassland region. It was considered that laboratory controlled experiments would indicate the relative activity of the microflora thus showing how these organisms have adapted themselves to the soils of this region. Since the characteristics of the soil habitat may influence the microflora present, some chemical analyses were carried out on each soil in order to relate these to the nitrogen-fixing activities found.

It is considered advisable to briefly describe the region from which samples were taken for this project.

Description of Region

As mentioned earlier, the grassland region of Saskatchewan was selected for this preliminary study. The brown, dark brown and black soil zones comprise the main portion of this region. The grassland formation extends from the southwest corner of the province to the edge of the forest or wooded region to the north and north east.

Vegetation

The region is divided into the short-grass prairie, the mixed prairie and the aspen-grove or parkland area. These divisions of vegetation correspond to broad variations in climate and soil moisture efficiency. They are also related to the major soil zones.

Climate

The natural vegetation and soil weathering processes have been affected by climate. The climate changes from a semi-arid condition in the southwest to a sub-humid climate in the northerly and easterly sections of the grassland region. Precipitation varies from 11 inches per year in the southwest to 18 inches in some areas of the black soil zone. Some parts of the more humid sections do not have much more precipitation than the southerly regions but, the evaporation rate being less, more efficient use can be made of the moisture present. The Cypress Hills area in the southwest, because of its altitude, has a higher precipitation than the surrounding prairie, making the vegetation comparable to the sub-humid section of the province.

Soil Zones

The brown soil zone in the southwest comprises the short-grass and part of the mixed prairie region. The dry climate of this area is reflected in the sparser vegetation. In the dark brown soil zone, slightly better moisture conditions prevail. This area comprises most of the mixed prairie type of vegetation. In the black soil zone, better moisture conditions are reflected in a heavier vegetation. This zone corresponds to the parkland vegetation region.

There is a general increase in nitrogen and organic matter of these soils as one moves in a northerly and northeasterly direction. Reactions of the well-drained soils range from about pH 6.0 - pH 8.0. The different zones also vary in their fertilizer requirements where moisture is not such a sharply limiting factor.

Further information concerning the soils and agricultural practices in this region may be obtained from Mitchell et al. (62) or the excellent "Guide to Farm Practice in Saskatchewan."

Plan of Experiment

The initial investigation was started in the summer of 1947, and soil samples were obtained from three areas close to Swift Current, Saskatchewan. Soil from plots which had been seeded down to Crested Wheat Grass for six years and adjacent virgin and cultivated soils were sampled. Composite samples were from the 0-6" layer, taken with sterilized equipment. These soils were air-dried in the laboratory and precautions

taken to prevent contamination.

The above soils were inoculated into suitable nitrogen free media in the laboratory, using replicates and sterilized controls. Total nitrogen was determined after incubation of such cultures. Soils were inoculated onto nitrogen free agar media in further studies. Solution cultures were examined for films, odors and pH values. Microscopic examination was made to determine presence of azotobacter cells and by inoculation onto suitable agar media.

In September 1947, some 68 soil samples were taken from the grassland region of Saskatchewan for nitrogen-fixation studies in the laboratory. Considerable experimental work was carried out to determine the most suitable methods for culturing these soils in the laboratory.

Work reported in this thesis on the above samples represents only the experiments conducted during the winter of 1950-51 when forty of the soil samples were studied. Locations 1-17 represent virgin and the adjacent cultivated soil from the three soil zones. Different soil textures and soil associations were represented. Location 18 represents virgin soil samples from the Cypress Hills. One sample is from the Lodgepole area and one from the golf course plain. Four soil samples were taken from a special area of the Indian Head Forest Nursery station. Difficulty had been experienced in obtaining stands of seedling trees in some parts of this area. Examination of these soils in the laboratory had revealed a

high content of nitrate where growth had been most seriously affected.

For the major portion of this project, white quartz sand was used as a base for culturing the soil inoculum. It was considered that sand would furnish aerobic conditions and facilitate distribution of the soil inoculum throughout the culture medium.

Some of the soils were inoculated into nitrogen-free solution cultures for comparison with the soil cultures, using various concentrations of sugar.

Activities of both nitrogen-fixing bacteria and other bacterial flora were studied in a few soil and sand cultures. This phase of study was carried out on a limited scale merely to compare conditions prevailing in artificial cultures and under natural soil conditions. Attempts were made to count numbers of azotobacter from the different cultures and, failing this, to at least determine if viable cells were present.

MATERIALS AND METHODS

Sampling

In all locations samples were taken at a depth of 0-6 inches. Previously sterilized quart jars were used for collecting the samples. Each sample represents a composite of four samplings from the immediate area. It was not possible at all times to use freshly sterilized tools (trowels) and in

these cases the tools were washed, then thoroughly scoured with the soil being sampled, before each sample was taken.

The above sealed samples were air-dried in sterilized pans in the greenhouse at the Soil Research Laboratory, Swift Current. Care was taken to prevent contamination between soils or from other sources. Samples were then returned to their original jars.

Grinding was accomplished in a hand grinder, steamed and dried between samples. Smaller samples were ground finely by mortar and pestle, sterilized between samples with 5% mercuric chloride. It is realized that perfectly aseptic conditions are difficult to maintain because of air contamination, but every precaution was taken to keep this at a minimum. All samples were tightly sealed and stored for future use.

Sand Cultures

Thoroughly washed, white sand, obtained from Ottawa, Illinois, was used in the main project, as a base for the culture medium. Fifty grams of the sand, in 300 ml. erlenmeyer flasks, stoppered with cotton, were used. Flask and contents were autoclaved at 15 pounds steam pressure for thirty minutes.

Each flask of sand was inoculated with 0.1 gram of soil and well distributed by gentle agitation. Freshly sterilized spatulas and weighing dishes were used between samples. By aseptic technique, 10 ml. of a sterile nitrogen-free solution was added to each flask.

Samples from one location, a virgin and a cultivated soil,

were set up in each case and incubated together. Twenty flasks were used for each soil, permitting four separate inoculation periods of 7, 14, 28 and 49 days. There were therefore five cultures for each incubation time, and two of these were immediately sterilized to be used as controls. All culture flasks were incubated in closed cupboards where the temperature range was from 23-26°C. during the experiment.

Flask weights were checked at intervals and it was found that there was a loss of about four grams by evaporation, after four weeks' incubation. Consequently all flasks were brought up to their original weights by adding sterile distilled water.

The solution medium used in the sand cultures is that described by Fred and Waksman (25), modified to contain the following constituents:

Mannite	$C_6H_8(OH)_6$ ----------	20.0 grams
Magnesium sulfate	$MgSO_4 \cdot 7H_2O$ ----	0.2 "
Dipotassium phosphate	K_2HPO_4 ------	0.2 "
Sodium chloride	NaCl ----------	0.2 "
Calcium sulfate	$CaSO_4 2H_2O$ --------	0.1 "
Calcium carbonate	$CaCO_3$ ----------	5.0 "
Distilled water	----------	1000.0 "

As 10 milliliters of the above solution were inoculated into each sand culture, 0.2 grams of mannite would be present in each flask.

Solution Cultures

A. Virgin, Crested Wheat Grass and Cultivated Soils

In this experiment, 100 ml. of the nitrogen-free solution described on the preceding page was sterilized in each cotton-stoppered 500 ml. erlenmeyer flask. Four of these flasks were inoculated with 5 grams of each soil by aseptic methods. Two of the flasks were then sterilized by adding 20 ml. of concentrated sulfuric acid. All flasks were incubated at 30°C. for 28 days and contents then transferred to Kjeldahl flasks for the nitrogen determination.

B. Soils from Locations 1 to 17 and Indian Head

A few of the soils used in the sand culture experiment were inoculated into solution cultures. The mannite content of the solution was adjusted so that 0.2 grams was present in each 100 milliliters of solution in 500 milliliter erlenmeyer flasks; 0.1 grams of soil was inoculated into each flask by aseptic methods. Replication and incubation were the same as with the sand cultures. Nitrogen determinations were made after suitable incubation.

Because of the low nitrogen fixation found in the above experiment, further work was begun using 50 milliliters of solution containing 0.2 grams of mannite or a 0.4% concentration. A few of the soils were also inoculated into culture solution containing 1 gram of mannite or 2.0% and flasks incubated for five weeks.

After 3 weeks' incubation there was no indication of nitrogen fixation in the flasks containing 0.2 grams mannite. It

was considered that the mannite concentration was too low and this was increased so that flasks now contained 1 gram or a 2.0% concentration. Flasks were incubated a further two weeks and contents of all transferred to Kjeldahl flasks for the nitrogen determination.

Bacteriological Examination

Attempts were made to determine numbers of azotobacter or at least isolate them, on nitrogen free agar. The above solution plus 1.5% agar was used as agar media.

Counts of total bacteria other than nitrogen fixing organisms were made from the various cultures, using sodium caseinate agar as described by Fred and Waksman (25).

In some cases, typical azotobacter colonies did not grow on the N-free agar plates. Solution cultures, which had been set up for all soils, were examined by microscopic methods for presence of the organism and agar streaks were made.

Analytical Methods

Total nitrogen was determined by the modified Gunning-Hibbard method, A.O.A.C. (65) making use of selenium as a catalyst. Sulfuric acid C.P. was used in the digestion and distilled water in the distillation to reduce errors from nitrogen impurities. The boric acid method of Scales and Harrison (71) was used for the recovery of ammonia in distillation. Boric acid 4% was used in the distillation and titrations made with N/50 sulfuric acid. For the nitrogen determinations on the original samples, N/14 sulfuric acid was used.

Total carbon determinations were made by the rapid

method of Walkley and Black (87). Easily soluble phosphorus was determined by the Truog method (79) as modified by Doughty (22). The extracting solution used in this method was 0.005 N sulfuric acid buffered to pH 3.0, by use of potassium acid phthallate. The Beckman spectrophotometer was used for the colorimetric measurements of the phosphorus.

Nitrate nitrogen was determined by the phenoldisulfuric acid method of Fred and Waksman (25) with colorimetric readings being made with the spectrophotometer.

The Beckman potentiometer was used for pH measurements, by the soil paste method as outlined by Doughty (21).

Figure 1

SOIL ZONES OF SASKATCHEWAN

LEGEND

- ● Location of Samples
- 0 Unsettled Area
- 1 Brown Soils
- 2 Dark Brown Soils
- 2A Mixture of Dark Brown, Brown and some Black Soils
- 3 Black Soils
- 4 Transition Soils
- 5 Grey Wooded Soils

RESULTS

In these experiments, a small amount of soil was inoculated into a medium containing other essentials for growth of nitrogen fixing bacteria. The soil would supply any trace elements necessary, but mainly it can only be considered as an inoculum containing the soil microflora. The nitrogen fixing ability of such an inoculum when placed under suitable conditions for growth, should reflect the suitability of such soil as a habitat for the nitroge-fixing microorganisms.

In Tables I and II some laboratory analyses of the original soils used in this project are presented. It is not a complete analysis, but covers a few soil constituents and properties shown by previous workers to affect the nitrogen-fixing microflora of the soil.

Soils from locations 1-17 in Table I show a pH range of 6.0-8.2 with an average of 7.3 for virgin soil and 7.6 for cultivated soils. The majority of these soils fall well within the range of pH 6.5-8.6 given by Fred and Davenport (24) as optimum for aerobic nitrogen fixing bacteria. Easily soluble phosphorus is present in all soils as shown by the method used for analysis, with little difference in the average content for virgin and cultivated soils.

The carbon content is about 23% higher on the average for the virgin than for the cultivated soils. A similar difference exists with nitrogen content, which averages 28% higher

for the virgin than for the cultivated soils.

The average results of analyses for each zone are shown at the bottom of Table I. Not enough soils, however, are represented in each zone to make significant comparisons possible. These results indicate greatest nitrogen and carbon losses by cultivation in soils of the dark brown zone. Brown (9) and others in extensive analytical data on soils from the three zones show that the greatest darbon and nitrogen changes by cultivation took place in the black zone.

Carbon-nitrogen ratios are lower generally than those reported by Brown (9) and by Mitchell et al. (62) for soils of this region. Differences in analytical methods used would likely account for this discrepancy.

Table II contains similar analytical data on soils from location 18 and four soils from the Indian Head Forest Nursery Station. Location 18 was in the Cypress Hills Park, one virgin soil being sampled in the lodgepole pine area and the other was from the golf course plain. The more humid conditions of this region are reflected in the lower pH, 5.0 and 5.6, of these soils. The easily soluble phosphorus content is quite low in these soils. The carbon and nitrogen contents of these soils are relatively higher than those of most soils sampled for this investigation.

The cultivated soils of the Forest Nursery Station are listed as (a), (b), (c) and (d) in Table II. Some differences

are noted in the pH; soil (c) is better drained and has a more favorable reaction than the others. Soil (a) has the poorest drainage of this sampling area. The nitrate nitrogen content is quite high (353 p.p.m.) as compared to soils (b), (c) and (d). Soil (a) also has the most acid reaction (pH 5.8) of this area. Difficulty had been experienced in obtaining seedling stands in the immediate area from which soil (a) was sampled. In earlier analyses at the Soil Research Laboratory, Swift Current, the author had found soils of this particular location to contain 800 p.p.m. of nitrate nitrogen.

Table III contains results of the initial studies made at Swift Current, Saskatchewan. Soil samples were from three locations where virgin, crested wheat grass, and cultivated soils were sampled. After incubating in tumblers for two weeks with and without 2.0% mannite at optimum moisture, 5 grams of each soil were inoculated into sterile solution cultures. The 100 ml. of solution in 500 ml. erlenmeyer flasks contained 2.0 grams of mannite (2.0% concentration). After incubation for 28 days and analysis for nitrogen, there are no consistent differences in amount of nitrogen fixed between virgin, crested wheat grass and cultivated soils from the three locations. More nitrogen was fixed in cultures by inoculating soils previously incubated with mannite.

TABLE I

Chemical Analyses of Original Soil Samples

Used for Nitrogen Fixation Studies (average of duplicate determinations)

Location	Zone	Soil Type	Virgin or Cult.	pH	Easily soluble P, p.p.m.	Carbon %	Nitrogen %	C:N ratio
1	Brown	Sceptre heavy clay	Virg.	7.7	22	2.55	.305	8.36
			Cult.	7.7	27	1.80	.222	8.12
2	"	" " "	Virg.	7.6	26	1.35	.230	5.87
			Cult.	7.7	27	1.40	.230	6.08
3	"	" " "	Virg.	8.0	38	1.26	.217	5.81
			Cult.	7.9	35	1.02	.180	5.66
4	"	Wood mountain loam	Virg.	6.9	11	1.80	.200	9.00
			Cult.	6.9	19	2.58	.285	9.05
5	"	" " "	Virg.	7.1	47	2.30	.295	7.79
			Cult.	7.3	6	2.04	.300	6.80
6	"	Haverhill clay loam	Virg.	7.9	19	1.40	.220	6.36
			Cult.	7.4	20	1.65	.195	8.46
7	Dark brown	Regina heavy clay	Virg.	7.9	27	2.07	.285	7.26
			Cult.	7.9	27	1.50	.235	6.38
8	" "	" " "	Virg.	6.9	12	2.64	.425	6.21
			Cult.	7.6	12	1.41	.210	6.71
9	" "	" " "	Virg.	7.7	25	2.20	.315	6.98
			Cult.	7.8	27	1.56	.235	5.38
10	" "	Elstow silt loam	Virg.	6.7	16	2.19	.315	6.95
			Cult.	7.4	24	1.95	.290	6.72

TABLE I (continued)

Location	Zone	Soil Type	Virgin or Cult.	pH	Easily soluble P, p.p.m.	Carbon %	Nitrogen %	C:N ratio
11	Dark brown	Elstow silt loam	Virg.	6.1	19	1.74	.245	7.10
			Cult.	7.3	16	1.65	.250	6.60
12	" "	Elstow silty clay loam	Virg.	6.0	19	5.85	.567	10.30
			Cult.	7.6	5	2.64	.300	8.80
13	" "	Weyburn loam	Virg.	7.8	29	1.44	.225	6.40
			Cult.	7.6	16	1.77	.265	6.68
14	" "	" "	Virg.	7.4	22	6.00	.595	10.08
			Cult.	7.4	14	3.40	.300	11.33
15	Black	Indian Head clay loam	Virg.	7.5	16	2.04	.290	7.03
			Cult.	8.2	19	2.52	.267	9.43
16	"	Yorkton light loam	Virg.	7.0	10	3.39	.375	9.04
			Cult.	7.4	15	2.60	.305	8.52
17	"	Oxbow light loam	Virg.	7.9	13	2.90	.395	7.34
			Cult.	8.0	31	2.10	.275	7.63
Average of brown zone (6 soils)			Virg.	7.5	27	1.77	.244	7.25
			Cult.	7.5	22	1.75	.235	7.44
Average of dark brown zone (8 soils)			Virg.	7.1	21	3.01	.371	8.11
			Cult.	7.6	18	1.98	.260	7.61
Average of black zone (3 soils)			Virg.	7.5	13	2.77	.353	7.85
			Cult.	7.9	21	2.40	.282	8.53
Average of above 17 locations			Virg.	7.3	22	2.53	.323	7.83
			Cult.	7.6	20	1.97	.255	7.72

TABLE II
Chemical Analyses of Original Soil Samples
Used for Nitrogen Fixation Studies (average of duplicates)

Location	Zone	Soil Type		pH	Easily soluble P, p.p.m.	Carbon %	Nitrogen %	C:N Ratio	N as NO_3 p.p.m.
18. Cypress Hills Park	Black	Cypress clay loam	*	5.0	2	4.65	0.520	8.94	-
			**	5.6	1	5.55	0.520	6.73	-
Indian Head Forest Nursery Station	Black	Indian Head clay loam	#a	5.8	12	2.59	0.305	8.49	353
			b	6.5	14	1.63	0.197	8.27	29
			c	6.1	15	1.62	0.187	8.66	12
			d	7.5	17	2.60	0.375	6.93	30

*Soil from lodgepole area.

**Soil from golf course area.

#a - Soil from area of poor seedling tree growth.

b - " " 40 yards west of (a), better growth.

c - " " 100 " " " (a), good growth.

d - " " 110 " " " (a), " " " better drained and beside Carragana hedge.

TABLE III

Nitrogen Fixed by Inoculating 5 Grams of Soil into 100 ml. of a 2.0% Mannite Solution and Incubated for 28 Days*

Location	Soil Type	Cultural Condition	Previous Treatment	Nitrogen in incubated flasks, (ave.) mgms.	Nitrogen in checks, (ave.) mgms.	Nitrogen fixed per gram of mannite mgms.	Azotobacter isolated
1	Haverhill loam	Virgin	Mannite	23.8	11.6	6.1	+
			No "	18.8	12.6	3.1	+
		Crested wheat grass	Mannite	22.6	9.4	6.6	+
			No "	17.6	10.7	3.4	+
		Cultivated	Mannite	19.9	7.9	6.0	+
			No "	15.6	9.1	3.2	+
2	Haverhill loam	Virgin	Mannite	23.6	10.2	6.7	+
			No "	20.3	11.7	4.3	+
		Crested wheat grass	Mannite	23.6	10.7	6.4	+
			No "	17.8	12.6	2.6	+
		Cultivated	Mannite	23.9	10.8	6.5	+
			No "	16.8	12.0	2.4	+
3	Sceptre clay	Virgin	Mannite	23.8	11.0	6.4	+
			No "	18.6	13.2	2.7	+
		Crested wheat grass	Mannite	23.9	9.5	7.2	+
			No "	15.7	11.6	2.0	+
		Cultivated	Mannite	23.9	8.4	7.7	+
			No "	19.0	8.3	4.1	+

Locations: 1. Experiment station proper
2. " " b (Sykes farm).
3. Near Beaver Flats (Cornelson farm).

*Soils were previously incubated with and without mannite for 2 weeks at optimum moisture condition.

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In Table IV results are presented for sand culture experiments conducted during the winter of 1950-51 on soils from Locations 1-17. Sand was used as a base in order to supply aerobic conditions somewhat similar to those obtaining in the field.

Triplicate results are given in order to show the degree of variation evident between cultures. Each figure represents the amount of nitrogen fixed per flask after deducting the amount present in the sterilized controls. Results are reasonably consistent considering that figures represent a measurement of biological activities.

The greatest variation between replicates exists where there has been lower fixation and some of this variation must be attributed to experimental error.

The amounts of nitrogen fixed vary from 0.0 to 11.5 milligrams per gram of mannite. In most cases there was no significant increase in nitrogen fixed after four weeks incubation. As shown by average figures at the bottom of Table IV, cultivated soils showed a slightly higher rate of nitrogen fixation in cultures and the final amount fixed in seven weeks is also a little higher, but these differences may not be significant.

TABLE IV
Nitrogen Fixed by Inoculating 0.1 grams of Virgin and Cultivated Soils into Sand Cultures containing 0.2 grams (2.0%) of mannite

Location	Zone	Soil Type	Milligrams Nitrogen fixed per gram of Mannite							
			7 days		14 days		28 days		49 days	
			(a)≠	(b)≠≠	(a)≠	(b)≠≠	(a)≠	(b)≠≠	(a)≠	(b)≠≠
1	Brown	Sceptre heavy clay	2.94	2.64	6.32	7.35	6.76	8.38	8.82	10.87
			2.94	1.47	4.85	7.05	7.93	10.44	7.06	9.41
			2.79	1.62	5.73	8.87	7.49	8.82	6.46	9.85
		Ave.	2.89	1.91	5.63	7.69	7.39	9.21	7.45	10.04
2	"	"	2.35	1.91	5.44	6.46	10.87	7.64	9.11	7.06
			3.23	1.76	6.90	5.29	10.29	6.02	7.06	7.79
			2.94	1.91	5.44	7.64	10.58	7.79	8.08	6.61
		Ave.	2.87	1.86	5.92	6.46	10.58	7.15	8.08	7.15
3	"	"	3.97	3.82	6.76	7.20	5.88	7.35	5.59	6.61
			3.67	3.97	6.76	7.50	5.73	6.32	6.47	5.88
			2.94	3.97	7.35	7.64	6.17	7.06	6.61	4.41
		Ave.	3.52	3.92	6.96	7.45	5.92	6.91	6.22	5.63
4	"	Wood Mountain loam	0.15	0.00	1.61	7.50	2.79	7.64	0.29	9.26
			0.00	0.44	1.02	4.41	3.82	8.82	1.47	8.38
			0.73	0.29	0.59	4.56	6.17	8.38	2.35	8.82
		Ave.	0.29	0.24	1.07	5.49	4.26	7.95	1.37	8.82
5	"	"	0.88	2.20	6.91	5.14	7.06	9.85	12.35	7.79
			1.32	2.20	5.73	6.03	7.79	7.64	8.97	7.35
			0.44	2.06	4.85	5.59	7.06	5.44	9.85	7.64
		Ave.	0.88	2.15	5.83	5.58	7.30	7.64	10.39	7.59
6	"	Haverhill clay loam	0.15	0.15	0.29	0.29	0.00	0.73	1.47	1.62
			0.44	0.29	0.29	0.44	-0.29	1.30	2.94	0.29
			0.59	0.59	0.59	0.29	0.44	-0.29	2.35	-
		Ave.	0.39	0.34	0.39	0.34	0.05	0.58	2.25	0.95
7	Dark brown	Regina heavy clay	0.88	4.56	2.50	7.35	3.09	5.29	4.56	5.73
			0.88	6.76	2.94	6.61	2.65	7.79	4.70	5.88
			0.88	4.41	3.23	7.64	2.06	6.91	5.14	6.03
		Ave.	0.88	5.25	2.89	7.20	2.60	6.66	4.80	5.88
8	"	"	2.50	2.65	5.59	3.38	4.56	6.20	5.00	4.70
			2.50	0.88	4.41	4.99	5.29	5.88	5.00	5.00
			2.20	1.76	5.88	5.29	5.00	6.03	4.41	4.12
		Ave.	2.40	1.76	5.29	4.55	4.95	6.03	4.80	4.60
9	"	"	0.59	0.88	2.50	4.56	6.91	5.00	7.94	1.76
			0.44	0.74	4.70	4.85	5.14	4.56	5.73	1.76
			1.03	0.44	4.40	5.59	7.50	5.73	6.47	2.05
		Ave.	0.69	0.69	3.86	5.00	6.52	5.09	6.71	1.86

TABLE IV (continued)

Location	Zone	Soil Type	Milligrams Nitrogen fixed per gram of Mannite: 7 days (a)≠	7 days (b)≠≠	14 days (a)≠	14 days (b)≠≠	28 days (a)≠	28 days (b)≠≠	49 days (a)≠	49 days (b)≠≠
10	Dark brown	Elstow silt loam	3.23	1.91	5.88	4.41	4.85	4.85	5.29	4.56
			1.47	2.94	6.17	4.26	4.85	4.26	5.14	4.26
			0.88	3.23	4.56	4.70	5.29	4.26	5.14	4.12
		Ave.	1.53	2.69	5.53	4.46	4.99	4.45	5.19	4.31
11	"	"	0.00	0.00	0.73	0.15	0.15	0.44	3.53	0.56
			0.00	0.15	2.05	0.29	3.97	3.82	0.73	0.42
			0.29	0.44	3.53	0.15	1.32	0.59	1.91	0.28
		Ave.	0.09	0.19	2.00	0.19	1.81	1.61	2.06	0.42
12	"	Elstow silty clay loam	0.00	2.49	2.06	10.40	1.47	9.41	0.59	10.10
			0.44	4.26	1.76	4.70	2.94	11.61	1.17	8.38
			0.73	4.56	1.91	5.14	2.35	13.52	1.47	12.35
			0.39	3.77	1.91	6.74	2.25	11.51	1.07	10.27
13	"	Weyburn loam	0.56	2.52	1.26	7.28	-0.10	6.02	1.26	0.84
			0.00	0.84	0.15	7.00	0.15	3.78	1.54	5.46
			0.84	2.66	0.42	6.86	0.00	7.42	0.98	6.16
		Ave.	0.47	2.01	0.61	7.05	0.02	5.74	1.26	4.15
14	"	"	0.98	1.54	3.78	4.48	2.66	10.22	2.20	7.79
			1.68	1.47	7.00	1.54	7.00	6.86	3.97	8.08
			1.47	1.54	3.92	8.54	5.32	5.18	4.70	6.61
		Ave.	1.38	1.52	4.90	4.85	4.99	7.42	3.62	7.49
15	Black	Indian Head clay loam	-0.29	0.88	3.38	4.70	6.47	7.35	8.82	4.56
			0.00	0.59	2.35	5.58	5.29	6.03	8.23	6.32
			1.17	1.62	1.62	4.56	7.64	4.99	8.23	10.44
			0.29	1.03	2.45	4.95	6.46	6.12	8.42	7.10
16	"	Yorkton light loam	0.10	2.94	0.73	8.08	8.82	9.85	1.18	6.03
			0.10	6.91	6.17	6.61	8.23	6.17	1.47	7.49
			0.10	4.99	1.62	9.41	7.64	7.49	1.76	7.20
		Ave.	0.10	4.95	2.84	8.03	8.23	7.84	1.47	6.90
17	"	Oxbow light loam	0.44	0.29	2.64	1.76	0.29	7.35	0.29	0.29
			0.59	0.44	0.88	1.62	0.15	2.79	1.32	0.44
			0.88	0.29	0.59	0.15	3.38	3.09	0.00	0.00
		Ave.	0.63	0.34	1.37	1.18	1.27	4.41	0.53	0.24
Average of 17 locations			0.86	1.36	4.97	5.13	4.68	6.25	4.45	5.47

(a)≠ Virgin
(b)≠≠ Cultivated

Table V contains results of sand culture studies on soils from Location 18 in Cypress Hills Park and soils from the Indian Head Nursery Station. The two soils from location 18 fixed very little nitrogen in sand cultures up to seven weeks of incubation. Amounts shown would likely be within the experimental error of the method, since losses of nitrogen are recorded in some of the replicates.

Very little nitrogen fixation took place in sand cultures inoculated with soil (a). Soils (b), (c) and (d) fixed about the same amount of nitrogen after seven weeks incubation. In all soils but (d) there was a higher fixation after seven weeks than after four weeks incubation. The total amount fixed is approximately the same as the average recorded for soils of locations 1-17 in Table IV.

TABLE V

Nitrogen Fixed by Inoculating 0.1 grams of Virgin and Cultivated Soils Into Sand Cultures Containing 0.2 grams (2.0%) of Mannite

Location	Zone	Soil Type	Milligrams Nitrogen Fixed per gram of Mannite							
			7 days	14 days	28 days	49 days	7 days	14 days	28 days	49 days
			*				**			
18-Cypress Hills Park	Dark brown	(Virgin) Cypress clay loam	0.00	0.59	0.00	0.29	0.15	0.29	0.59	-
			0.00	0.73	0.00	0.29	0.73	0.00	0.73	0.59
			-0.10	0.73	0.29	0.59	0.29	0.15	0.59	0.29
			0.03	0.68	0.09	0.39	0.39	0.15	0.64	0.29
		(Cultivated)								
Indian Head Forest Nursery Station	Black	Indian Head clay loam	(a)				(b)			
			0.44	0.88	-1.03	1.62	0.00	2.94	4.70	7.35
			1.17	2.06	1.91	0.73	0.29	4.26	5.73	4.41
			0.44	1.47	1.32	2.35	0.29	2.50	6.32	5.88
			0.68	1.47	0.73	1.56	0.19	3.23	5.58	5.88
			(c)				(d)			
			0.15	1.76	3.67	3.82	1.47	2.20	0.29	8.38
			0.29	5.40	3.23	3.97	0.73	3.82	1.76	6.17
			0.88	2.90	2.79	4.26	1.03	1.91	2.49	3.82
			0.44	3.35	3.23	4.02	1.08	2.64	1.51	6.02

*Soil from lodgepole area.
**Soil from golf course area.

(a) - Soil from area of poor seedling tree growth.
(b) - " " 40 yards west of (a), better growth.
(c) - " " 100 " " " (a), good growth.
(d) - " " 110 " " " (a), good growth, better drained and beside Carragana hedge.

Table 7

Difference Between Outstanding Debt and Unexpended Bond Proceeds
Selected Cities in Texas (000's) and Per Capita Amounts

Results of inoculating some of the soils, used in experiments reported above, into solution cultures are given in Table VI. As with sand cultures 0.1 grams of soil were inoculated into each 500 ml. flask containing 100 ml. of sterile nitrogen-free mannite solution. Each flask contained 0.2 grams as did sand culture flasks. Very little fixation took place in such cultures after incubating up to seven weeks. No characteristic films or odors were noticeable in any of the flasks after seven weeks.

An experiment was begun later using 50 ml. of sterile nitrogen-free mannite solution in 300 ml. flasks. The same amount of soil (0.1 grams) was inoculated into each flask which contained 0.2 grams of mannite at a concentration of 0.4 percent. It was intended to keep the amount in each flask the same but to double the concentration. Similar flasks, but containing 1 gram of mannite or 2.0% concentration, were also inoculated with a few of the same soils.

Flasks containing 0.4% concentration of mannite failed to show signs of nitrogen fixation by film formation or odors after 3 weeks. It was concluded that no fixation was taking place. Mannite was then added to bring the concentration up to 2.0% and flasks were incubated a further two weeks.

TABLE VI

Nitrogen Fixed by Inoculating 0.1 grams of Virgin and Cultivated Soils into 100 ml. of a Nitrogen-free Solution Containing 0.2 grams (0.2%) Mannite

Location	Zone	Soil Type	Milligrams Nitrogen fixed per gram of Mannite							
			7 Days		14 days		28 days		48 days	
			Virgin	Cult.	Virgin	Cult.	Virgin	Cult.	Virgin	Cult.
1	Brown	Sceptre heavy clay	0.00	0.00	0.15	0.00	0.15	0.15	0.69	0.00
			0.00	0.15	0.73	0.15	0.15	0.00	1.22	0.15
			0.59	0.00	0.59	0.00	0.38	0.15	0.52	0.29
		Ave.	0.19	0.05	0.49	0.05	0.23	0.10	0.81	0.15
2	"	Sceptre heavy clay	1.17	0.44	1.32	0.44	0.15	0.15	0.44	0.44
			0.59	0.59	1.17	0.58	0.15	0.29	0.59	0.29
			0.88	0.59	2.20	0.73	0.00	0.29	0.15	0.00
		Ave.	0.88	0.54	1.59	0.55	0.10	0.24	0.39	0.24
9	Dark brown	Regina heavy clay	0.44	0.59	0.15	0.00	0.15	0.15	0.44	0.44
			0.29	0.44	0.73	0.29	0.29	0.44	0.00	0.15
			0.15	0.29	0.59	0.29	0.15	-	0.15	0.29
		Ave.	0.29	0.44	0.49	0.19	0.19	0.19	0.19	0.29

Results in Table VII, with the exception of the Leduc soil, are calculated on the basis of 1 gram of mannite being present per flask. Direct comparison can only be made by assuming that there was no appreciable fixation for 3 weeks while only 0.4% concentration was present. The two series can then be compared on the basis of the amount fixed for 2 weeks and 5 weeks with 2.0% mannite present.

It can be seen that amounts fixed per gram of mannite were lower in the solution cultures than in the sand cultures inoculated with the same soils. It was noted that where 2.0% mannite was present, film and odors were present in flasks after 2 weeks incubation.

The Leduc soil used for comparative purposes, fixed more nitrogen than the other soils used in solution cultures. More nitrogen was fixed per gram of mannite in flasks having a concentration of 0.4% mannite than in flasks with 2.0% mannite incubated for the same time.

TABLE VII

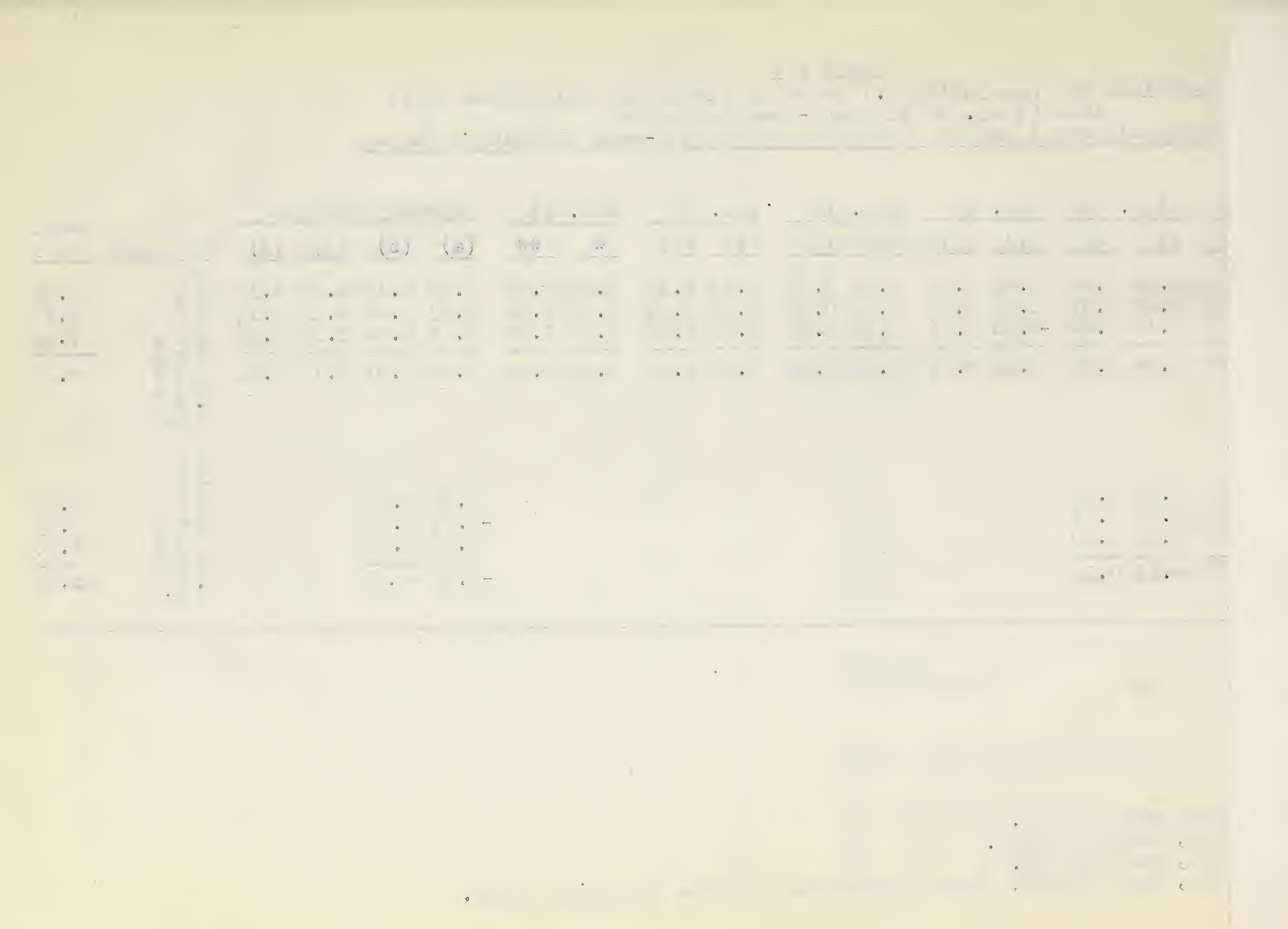
Nitrogen Fixed by Inoculating 0.1 grams of Virgin and Cultivated Soils into 50 ml. of Nitrogen-free Solutions Containing Different Levels of Mannite During a 5-week Incubation Period

Culture Treatment	Loc. 1		Loc. 2		Loc. 11		Loc. 12		Loc. 14		Loc. 17		Loc. 18		Nursery Station				Treatment	Leduc soil
	(1)≠	(2)#	(1)	(2)	(1)	(2)	(1)	(2)	(1)	(2)	(1)	(2)	*	**	(a)	(b)	(c)	(d)		
0.4% mannite present for 3 weeks + 2.0% for 2 additional weeks	3.06	0.28	3.20	0.73	0.31	0.14	0.07	0.38	0.31	2.78	0.17	0.35	0.32	0.00	0.07	0.10	0.21	0.17	0.4% mannite present for 5 weeks	11.65
	1.81	0.12	0.31	0.45	0.48	0.03	0.14	0.07	0.24	0.22	0.17	0.31	0.27	0.00	0.03	0.07	0.14	0.14		9.25
	2.02	0.69	0.25	3.27	0.17	0.10	-0.10	0.27	0.31	1.21	0.21	0.28	0.32	2.68	0.10	0.00	0.10	0.32		7.45
Ave.	2.29	0.36	0.75	1.48	0.32	0.09	0.04	0.24	0.28	1.40	0.18	0.31	0.30	0.89	0.06	0.01	0.15	0.21		9.45
2.0% mannite present for 5 weeks			3.90	2.82	0.10	0.35									0.00	-0.10			2.0% mannite present for 5 weeks	7.03
			4.10	4.21	0.17	0.17									-0.07	0.03				5.63
			4.10	3.86	0.10	0.00									0.00	0.03				6.40
Ave.			4.00	3.63	0.12	0.14									-0.02	-0.01				6.35

≠Virgin
#Cultivated

*Soil from lodgepole area.
**Soil from golf course area.

(a) Soil from area of poor seedling tree growth.
(b) " " 40 yards west of (a), better growth.
(c) " " 100 " " " (a), good growth.
(d) " " 110 " " " (a), good growth, better drained and beside Carragana hedge.



. The activity of bacterial microflora other than that of nitrogen-fixing bacteria was studied briefly in this project. Table VIII compares the amount of nitrogen fixed in sand cultures after four and seven weeks with total numbers of bacteria in similar cultures and in natural soil incubated at optimum moisture.

There was very little increase in nitrogen fixation in sand cultures after four weeks incubation. In similar cultures of the same soils, it was found that the numbers of bacteria that would grow on sodium caseinate agar were less at 7 weeks in all cases but one than at 4 weeks. The numbers of bacteria in sand cultures were higher generally than in natural soils incubated for the same length of time. Differences are not consistent, however.

Plate counts to determine total numbers of bacteria were made on a few soils that had shown differences in nitrogen fixation in cultures. Results of such counts are shown in Table IX. The soils had previously been incubated in tumblers with 2.0% mannite for four weeks. Attempts were also made to count numbers of azotobacter on nitrogen free agar, from the various cultures. Results obtained were not considered reproducible and were not recorded. It was, however, possible to isolate azotobacter from cultures of all soils except two from location 18. It will be recalled that these soils had reactions of pH 5.0 and 5.6.

TABLE VIII
Nitrogen Fixed in Sand Cultures,
Total Numbers of Bacteria in Sand Cultures
and Untreated Soil Cultures after Four
and Seven Weeks Incubation

Location	Virgin or Cult.	Milligrams Nitrogen fixed per gram of mannite		Total Numbers of bacteria per gram of sand culture (millions)		Total Numbers of bacteria per gram of soil (millions)	
		4 weeks	7 weeks	4 weeks	7 weeks	4 weeks	7 weeks
1	Virgin	7.39	8.45	8.12	5.72	6.5	9.0
	Cult.	9.21	10.04	6.14	26.03	12.0	14.5
2	Virgin	7.15	8.08	34.25	1.50	13.0	16.0
	Cult.	8.08	7.15	42.86	7.30	15.1	14.5
9	Virgin	6.52	6.71	32.48	15.76	15.5	19.5
	Cult.	5.09	1.86	43.22	21.48	5.0	6.5

TABLE IX
Comparison of Bacterial Numbers
After Incubating Some Soils with 2.0% Mannite
in tumblers for four weeks

Location	Zone	Soil Type	Numbers of bacteria per gram of soil (millions) Virgin	Cult.
11	Dark Brown	Elstow silt loam	13.00	14.00
12	" "	Elstow silty clay loam	23.25	5.50
14	" "	Weyburn loam	5.50	7.50
17	Black	Oxbow light loam	9.00	12.75
Cypress Hills 18	"	Cypress clay loam	* 3.10 ** 8.25	
Forest Nursery Station	"	Indian Head clay loam		(a) 3.00 (b) 2.35 (c) 5.75 (d) 23.10

*Soil from lodgepole area. **Soil from golf course area.

DISCUSSION

The analytical data recorded in Table I would indicate that conditions prevailing in most of the soils studied here are suitable for a nitrogen fixing microflora. The presence of easily soluble phosphorus and the suitable reaction (pH 7.5 on the average) would favor nitrogen fixing activity if the organisms are present.

It would be difficult to say how much if any of the easily soluble phosphorus recorded here is available to the microflora of these soils. Methods used by different workers for determining easily soluble phosphorus vary considerably. Ziemiecka (91) found nitrogen fixing organisms inactive where soluble phosphorus content was below 10 parts per million. The limiting concentrations given by various workers vary considerably. The main difference between the virgin and cultivated soils is in the carbon and nitrogen content. These elements are lower in the cultivated than in the virgin soils.

In Table III no significant differences were noted between amounts of nitrogen fixed in solution cultures inoculated with crested wheat grass, virgin and cultivated soils. However, soils previously incubated with 2.0% mannite fixed more nitrogen than soils incubated in their natural state before being inoculated into solution cultures. This would indicate that a larger population of nitrogen-fixing microorganisms was present in such soils. It was possible to isolate azotobacter

from all cultures and there was film formation. After 28 days incubation the pH was down to 6.0 in most cultures indicating anaerobic bacterial activity also. It is difficult to say how much fixation in solution cultures is by anaerobic and how much by aerobic microorganisms.

Soils from locations 1-17 fixed different amounts of nitrogen in sand cultures. It was not possible to relate amounts fixed to the composition of the original soils. Very little difference on the average is noted between the virgin and cultivated soils in fixation of nitrogen. A slightly higher rate and amount of fixation by cultivated is indicated, but probably could not be considered as significant. Viable azotobacter organisms were isolated from all the soils from locations 1-17.

These results are not in agreement with results obtained by Martin (58) and by Greaves (36) who found that very few virgin soils studied contained azotobacter and fixation by such soils was much lower than by cultivated soils. No consistent relation between differences in fixation and easily soluble phosphorus, or pH, can be seen in these soils. However, in location 18 where the pH was 5.0 and 5.6, azotobacter were absent and no fixation took place in solution or sand cultures.

More nitrogen was fixed in sand cultures than in solution cultures when inoculated with the same soil, even where the same concentration of mannite was present. Soils showing little fixation in sand cultures fixed even less in solution cultures.

In Table V a high nitrate content (353 p.p.m.) in soil (a) appears to have been toxic to the nitrogen fixing flora of

the soil. There was little fixation by this soil in either sand or solution cultures. In nearby soils (b), (c) and (d) the nitrate content was low and there was a fair amount of fixation of nitrogen in sand cultures. Gainey (30) found that concentrations of nitrate much less than present in soil (a) were toxic to aerobic nitrogen fixing microorganisms. The fact that this concentration had been toxic to seedling trees, would substantiate this conclusion. It was possible to detect typical azotobacter cells by microscopic examination of cultures of this soil.

It would appear from Table VII that concentration of mannite in solution cultures has an effect on nitrogen fixation by the Saskatchewan soils studied. The Leduc soil, on the other hand, must have a very active nitrogen fixing flora or species more suitable to such cultures. There was considerably more fixation in solution by this soil. More nitrogen was fixed per gram of mannite where the concentration was 0.4% than where it was 2.0% in the solutions. This agrees with Waksman and Starkey (86), page 109, where they show that azotobacter fix more nitrogen per gram of mannite when lower concentrations are present.

Sand culture conditions of this experiment would also appear to be suitable to bacterial activity other than non-symbiotic nitrogen fixing organisms. Table VIII shows that larger numbers of bacteria were present in sand cultures generally, than in natural soil incubated for the same length of time at optimum moisture.

Numbers of bacteria growing on sodium caseinate agar did not increase after 4 weeks in sand cultures nor was there an increase in nitrogen fixed. This would indicate a possible relation between nitrogen fixation activity and activity of other bacteria. Winogradsky (89) suggested that the nigrogen fixing microorganisms use only the by-products of other microbial activity for their energy. Such a relationship may obtain in these cultures.

In sand cultures, it was noticed that very little nitrogen was fixed after four weeks' incubation. This would indicate that most of the mannite had been used up at this time. The fact that differences in final amount fixed exist, suggests that the microflora of the various soils differ in efficiency and numbers. Such differences in microflora are likely to be a reflection of the suitability of the natural soil habitat for supporting the nitrogen fixing flora.

Sand vs. Solution Cultures

Results from this investigation have indicated that sand cultures are more suitable than solution cultures for studying the nitrogen-fixing flora of the Saskatchewan grassland soils. Results between replicate flasks were more uniform in sand cultures than in solution cultures. Sand cultures were much easier to transfer and to handle generally.

In general, the greater fixation in sand cultures where conditions are relatively aerobic would indicate that the aerobic forms of nitrogen fixers are more active in these soils than the anaerobic.

The surface area exposed to the atmosphere in sand cultures is considerably higher than when solution cultures are used.

It would be erroneous to conclude that all fixation in sand cultures is by aerobic microorganisms, without further investigation. Possibly a symbiotic relation existed as suggested by Winogradsky, and quoted by Lind and Wilson (52). The anaerobic nitrogen fixers are able to grow because aerobic microorganisms make conditions somewhat anaerobic in parts of the culture.

Measurement of nitrogen fixation after several different incubation periods would seem advisable in both sand and solution culture studies. In this way rate of fixation as well as total amount of fixation would be measured. In further studies it would also be advisable to measure the amount of energy consumed after different incubation periods, in order to better determine the efficiency of the nitrogen-fixing organisms of the soil.

Practical considerations

It is possible that nitrogen-fixing flora of natural soils may not react favorably when inoculated into artificial culture media. Considerable experimental work would be required to determine how the various nitrogen fixing microorganisms respond to particular conditions of the experiment.

It is extremely difficult to obtain evidence showing how much nitrogen is fixed in natural soils by the free-living nitrogen-fixing organisms. The evidence from these experiments

shows that the aerobic nitrogen-fixing bacteria are more important than the anaerobic form in soils of the grassland region of Saskatchewan. While it is not possible to state from these experiments just how much nitrogen is added to the soil each year under field conditions, one can say that such microflora are present and active under the conditions of this experiment.

Any farm management practices which tend to favor the above conditions should increase the amount of nitrogen added to the soil by aerobic nitrogen fixing microorganisms.

SUMMARY

1. The nitrogen-fixing powers of virgin and cultivated soils from the grassland region of Saskatchewan have been investigated in the laboratory.

2. Sand cultures were found to be more suitable than solution cultures for studying the nitrogen-fixing flora of these soils.

3. The fact that aerobic conditions existing in sand cultures favored nitrogen fixation, indicates the importance of the aerobic non-symbiotic nitrogen-fixing organisms in these soils.

4. Viable azotobacter were isolated from all but two soils. These were acid soils of pH 5.6 and 5.0.

5. Rate and amount of nitrogen fixed was similar in sand cultures inoculated with cultivated and virgin soils.

6. Soil containing a high nitrate content (353 p.p.m.) fixed very little nitrogen in sand or solution cultures indicating a toxic effect on the nitrogen-fixing flora.

7. Some soils appeared to be more efficient than others in utilization of the energy present in cultures.

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11. [illegible] [illegible]

12. [illegible] [illegible]

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